Taxonomic summary of *Rhizoclosmatium* and description of four new *Rhizoclosmatium* species (Chytriomycetaceae, Chytridiales)

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ABSTRACT

Rhizoclosmatium globosum, the type for the genus *Rhizoclosmatium*, is commonly isolated from freshwater aquatic systems, especially on chitin-containing substrates. The genus *Rhizoclosmatium* currently includes three additional described species: *R. aurantiacum* and *R. hyalinum*, which can grow on chitin, and *R. marinum* reported growing on a marine green alga. Our study employed analyses of ribosomal gene sequences, zoospore ultrastructure, and thallus morphology and development to identify chytrid strains related to *Rhizoclosmatium*. Phylogenetic analyses of ribosomal genes of strains revealed three major lineages within a clade that includes the type, *R. globosum*. Based on these results, we describe four new species and one new variety of *Rhizoclosmatium,* all capable of growth on chitin: *R. sparsum, R. umbonatum, R. umbonatum* var. *sphaericum, R. persicum,* and *R. pessaminum*. We have also found that *R. globosum* strains can grow on a range of substrates, including chitin, cellulose, keratin, and pollen. This information is herein assimilated into the existing taxonomy of the genus, which is also summarized and nomenclaturally updated. Published on-line www.phytologia.org *Phytologia 101(2): 139-163 (June 21, 2019).* ISSN 030319430.

KEY WORDS: *Rhizoclosmatium sparsum, R. persicum, R. pessaminum, R. umbonatum,* cellulose, chitin, chytrid, Chytridiomycota**,** keratin, morphology, phylogeny, pollen, systematics, ultrastructure, zoospore.

Chitin is the second most abundant organic compound in nature (Keyhani and Roseman, 1999), and understanding its turnover is critical to understanding parts of the carbon and nitrogen cycling in aquatic ecosystems (Gooday, 1990). Turnover of chitin polysaccharides in marine water columns and sediments is attributed primarily to bacteria (Gooday, 1990; Keyhani and Roseman, 1999). The identity of the microbes degrading chitin in freshwater systems is less well studied, but it is known that it includes fungi, particularly chytrid fungi (Gooday, 1990; Sparrow, 1960). The role of chytrids in aquatic food webs and in nutrient recycling has recently been emphasized (Kagami et al., 2007a, 2007b, 2011, 2014, 2017; Rasconi et al., 2014). Thus, knowledge of the biodiversity of chitinophilic chytrids is fundamental to understanding nutrient cycling in aquatic systems.

In an early study of aquatic fungi on insect exuviae, Petersen (1903) highlighted chitin-containing materials as substrates for chytrids. In his study of inhabitants of insect exuviae, he erected the genera *Rhizoclosmatium* and *Siphonaria* and observed *Obelidium*. Interestingly molecular phylogenetic analysis of a broad range of chytrid taxa (James et al., 2006) revealed that *Rhizoclosmatium globosum*, the type of the genus, forms a distinct clade with four other genera (*Obelidium, Siphonaria, Podochytrium* and *Phlyctorhiza)*, all of which utilize chitin as a substrate. *Rhizoclosmatium globosum* is one of the most commonly reported chytrids isolated from freshwater aquatic habitats (Sparrow, 1960). Phylotypes of *Rhizoclosmatium* sp. have even been detected unexpectedly in municipal drinking water (Otterholt and Charnock, 2011). Its prevalence has been demonstrated from complementary isolation, culture, and

environmental sequence studies (Davis et al., 2013, 2016, 2018; Lefèvre et al., 2012). These studies also revealed molecular divergence of strains with morphologies somewhat similar to *R. aurantiacum*, suggesting greater species diversity than currently recognized (Davis et al., 2016) and highlighting the need for greater study of the genus.

Herein we summarize the taxonomy of the genus *Rhizoclosmatium* and explore the relationships of strains in culture, including *R. globosum* and unidentified species that can be placed in this genus based on molecular phylogenetics and morphology. In addition to morphology, we present zoospore ultrastructure and analyses of ribosomal gene sequences. From these analyses we establish four additional species and one variety in this genus.

MATERIALS AND METHODS

Culture and morphological observations: Collecting sites and isolation baits for strains included in this study are listed in Table 1. Strains brought into pure culture were maintained on PmTG agar (1 g peptonized milk, 1 g tryptone, 5 g glucose, 10 g agar, 1 L distilled water). After growth for seven days on PmTG agar, strains were flooded with sterile distilled water for 15-30 min., which resulted in discharge of zoospores. We inoculated PmTG agar and natural substrates (sweet gum pollen, chitin from shrimp exoskeletons, cellulose strips from onion bulb scale epidermis, and keratin from molted snake skin) with zoospore suspensions, incubated at 22° C, and made light microscopic observations of strains over a 1-5 day period using either a Nikon Eclipse E200 or Zeiss Axioskop microscope equipped with bright field, phase contrast and Nomarski interference contrast optics.

DNA extraction, purification, amplification and sequence alignment: After growing strains for 3–5 days in PmTG broth on a rotary shaker, we extracted and purified genomic DNA as previously described (Davis et al., 2013). Complete ITS1-5.8S-ITS2 (ITS) and partial large subunit RNA (LSU; 28S rDNA) genes were amplified with primer pairs: ITS5/ITS4 and LROR/LR5 respectively (Rehner and Samuels, 1994; Vilgalys and Hester 1990; White et al., 1990). Following PCR reactions (Davis et al., 2013), amplicons were purified with a Nucleospin Extraction II kit (Macherey-Nagel, Inc., Bethlehem, PA) and sequenced by Macrogen USA (Rockville, MD). We assembled the resulting sequences with Sequencher 4.5 (Genecodes) as previously described (Vélez et al., 2011). Our study includes 16 newly recorded 28S rDNA sequences for strains (Table 1) and eight new ITS sequences for the following strains: JA 20=MK314726; MB 07=MK314722; MB 10=MK314723; MB 48=MK314727; MP 49=MK314724; MP 73=MK314728; WB 219=MK314725; WB 236B=MK314729.

Molecular phylogenetic analyses: We used newly generated 28S rDNA sequences along with sequences downloaded from GenBank as shown in Table 1. Sequences were aligned with Clustal X (Thompson et al., 1997) and manually adjusted in BioEdit (Hall, 1999). Maximum parsimony (MP) trees were generated using PAUPRat (Sikes and Lewis, 2001), and bootstrap support values were generated from heuristic searches with 500 replicates, each with 10 random-stepwise addition replicates. Maximum likelihood (ML) phylogenies were constructed using GARLI 0.951 (Zwikl, 2006) as explained in Vélez et al. (2011). The best model of base substitution was selected using Modeltest 3.7 (Posada and Crandall, 1998). The best tree was obtained from 100 best tree searches using the HKY model of nucleotide substitution. Bootstrap values were calculated using 500 nonparametric replicates with the same substitution model. Trees were rooted with strain BR 097, *Chytriomyces hyalinus* (Vélez et al., 2011 show species identification of BR 097 is not *Chytriomyces (Chytridium) confervae*). Sequence similarities among strains of interest were calculated by pairwise alignment in BioEdit.

Zoospore ultrastructure: Zoospore ultrastructural organization of the following strains was examined: ATCC 22918, MP 44, MP 67, WJD 111, and WJD 185. Zoospore suspensions were fixed with 2.5% glutaraldehyde in 0.1 M sym-collidine buffer for 1 hr. at 21° C, washed three times in 0.1 M buffer, and

secondarily fixed with 1.0% osmium tetroxide in 0.1 M buffer for 1 hr. at 21° C in the dark. Following three washes with deionized water, zoospores were centrifuged at ~ 3× *g* and then infused with molten agar. After solidification, material was cut into blocks (~ 0.1–0.2 cm^3) and stained overnight in saturated aqueous uranyl acetate at 5° C. Material was then processed for electron microscopy as previously described (Powell et al., 2013). Stained sections were observed at 60 kV on a Hitachi 7650 transmission electron microscope (TEM).

RESULTS

Phylogenetic analysis: The dataset had 822 characters, of which 103 were parsimony informative. Our MP (L = 177 steps) and ML (-lnL = 693.51) phylogenetic assessments were identical, and the MP tree is presented (Fig. 1). In the MP tree, strains identified based on morphology as *Rhizoclosmatium* species place into three major well-supported lineages (Fig. 1, lineages A, B, C).

Lineage A with ≥99% support includes a clade (A1) of 29 *Rhizoclosmatium globosum* strains (≥99% support) and a sister clade (A2) of four strains (≥92% support) morphologically distinct from *R. globosum* and other described *Rhizoclosmatium* species. Cultures of strains in lineage A range in color from cream, light tan to rose-white. Within the *R. globosum* clade (A1), 28S rDNA gene sequence similarity ranges from 99.8-100%; ITS gene sequence similarity ranges from 99.3-100%. For example, the ITS sequence in strain JEL 347h (AY997076) is 100% similar to that of strain MB 49 (MK314724); 99.6% similar to that of strains WB 236B (MK314729) and MP 73 (MK314728); and 99.3% similar to that of strain MB10 (MK314723).

Divergence in the 28S rDNA and ITS genes distinguishes the two sister clades in Lineage A, justifying a new species. There is 98% 28S rDNA sequence similarity between strain JEL 347h (A1) and strain MP 56 (A2), and only 91.2% ITS sequence similarity between strain JEL 347h (*R. globosum* AY997076) and strain MP 56 (JX905553). In contrast within clade A2, there is greater ITS similarity, 99.8% similarity between strain MP 56 (JX905553) and strain MP 46 (JX905552) and 100% sequence similarity with strain WB 266C (JX905558). This is a range of ITS differences similar to that found in the *R. globosum* clade (A1).

Lineage B with ≥81% support (Fig. 1) is sister of lineage A and includes five strains in two clades (B1, B2). Cultures of strains in lineage B range in color from bright orange (JEL 128) to light orange (MP 44). Strains in lineage B have 95% 28S rDNA sequence similarity with JEL 347h *R. globosum* of lineage A. Thalli in the two clades of lineage B share the feature of an umbo on developing thalli from the time of zoospore encystment to zoospore discharge. Lineage B strains MP 44 (B1) and JEL 128 (B2) are 99.25% similar in their 28S rDNA sequences, but thallus differences support the two clades as varieties of a new species, one from Alabama (B1) and the other from Maine (B2).

Lineage C with ≥90% support (Fig. 1) consists of two well-supported clades (C1, C2), each with 100% support, and is sister of lineages A + B. Lineage C is molecularly divergent from lineage A; the 28S rDNA sequence in strain MP 67 (C1) is 89.3% similar to that of JEL 347h (A1). Cultures of strains in lineage C are orange. Five strains of a new species are in a clade (C1) sister of two strains of another new species (C2). Strain MP 67 (C1) is 93.3 % similar in its 28S rDNA sequence to that of the two strains in the sister clade (C2=JEL 823, JEL 849). Morphology and molecular sequence differences support two new species within lineage C.

Zoospore ultrastructure: The zoospore is elongate (Fig. 2A) and exhibits features consistent with a Group 1-type zoospore (Barr, 1980; Barr and Hartman, 1976). A cell coat covers the body of the zoospore (Fig. 2G) but not the flagellum. A thin, biconcave flagellar plug lies in the transition region of the axoneme (Fig. 2A). A veil is adjacent to the non-flagellated centriole (Fig. 2D). The kinetosome-

associated structure (KAS) consists of two sets of stacked plates, each at the side of the kinetosome (Fig. 2C, D). Microtubules are bundled as a microtubule root, which extends from the side of the kinetosome and passes between the KAS plates toward the microbody-lipid globule (MLC) fenestrated cisterna (Fig. 2I). Stacked cisternae of the Golgi apparatus are in the posterior end of the zoospore near the microtubular root, and one cisterna is partitioned (Fig. 2H).

The nucleus is partially inserted into the ribosomal aggregation (Fig. 2A, B) in the orange-pigmented *R. persicum* (strain MP 67) and *R. umbonatum* (strain WJD 185), but could be partially inserted or outside the ribosomal aggregation in zoospores of the hyaline to pink strain *R. globosum* (WJD 111). The MLC typically includes a single lipid globule, adjacent microbody and fenestrated membrane cisterna, and proximal mitochondria (Fig. 2C, E, F, J). Mitochondria are positioned between the binding ER and the ribosomal aggregation (Fig. 2A). A large paracrystalline inclusion (Fig. 2K, L) lies in the peripheral cytoplasm (see Letcher and Powell 2014 for definition).

Morphology and Habitat: Strains differ in color in culture, and the pigmentation can change with the age of the culture. Strains in lineage A are lighter, pinkish white to light tan. Strains in lineage B are darker than those in lineage A, and are light orange (MP 44, WJD 185) to dark orange (JEL 128, JEL 796). Strains in lineage C are bright orange. Although all strains can grow on chitin, strains can use a variety of substrates in addition to chitin, including cellulose, keratin, and pollen (Table 1). Strains of *Rhizoclosmatium* were found in a range of aquatic habitats including ponds, bogs, fens, vernal pools, lakes and rivers often in samples containing aquatic or bog vegetation (Table 1).

TAXONOMY

Rhizoclosmatium currently includes four described species, three as saprotrophs on pollen and chitin-containing substrates and one as a marine algal parasite (Sparrow, 1960). Herein we expand the number of species in *Rhizoclosmatium* and confirm shared morphological features for the genus. The thallus is monocentric, with globose to subglobose sporangia, and a rhizoidal system emerges from a subsporangial apophysis that is variously shaped (Figs. 3B; 4G, I; 5H, I; 6I; 7B, C, F; 8D). Tips of rhizoids are pointed (Fig. 8D, G), and a domed-shaped septum separates the rhizoidal system from the sporangium (Figs. 4K; 7F; 8G, H). During development of the incipient sporangium from the encysted zoospore, the primary nucleus (Antikajian, 1949) enlarges before mitosis takes place (Figs. 4I; 5K; 7B) and divides only after the sporangium has achieved its mature size. Zoospores escape through a single inoperculate discharge pore (Figs. 4K; 5M; 7D, E, F) or papilla into a vesicle where they swarm as a mass before swimming away (Figs. 3H; 4J; 5M; 7E; 8F, G). The position of the discharge pore varies from apical, subapical, lateral (Figs. 3F, G; 4K; 5L, M; 6J; 7D, E, F; 8G) to basal. Sparrow (1937) illustrates and describes basal discharge in *R. aurantiacum,* but we did not observe basal discharge in any of our orange strains in culture. Consequently, we do not consider any of our orange strains as *R. aurantiacum*. Zoospores are posteriorly uniflagellate and vary in shape from elongate, oval to spherical (Figs. 3A; 4A; 5A; 6A; 8A). Resting spores when present are spherical and epibiotic. Sparrow (1937) reported sexual reproduction by rhizoidal conjugation of contributing thalli, but we did not observe rhizoidal conjugation in any of the strains studied. Much variation in rhizoidal complexity and sporangial and apophysis shape is found in these species. Below we summarize the current species, describe four new species and one variety, and provide a key to species of *Rhizoclosmatium*.

Rhizoclosmatium H. E. Petersen, Journ. de Botanique 17:216, 1903.

Mycobank MB 20480

Typification: Rhizoclosmatium globosum H. E. Petersen 1903 (TYPE SPECIES)

Description: Thallus consists of a spherical to variably shaped sporangium, with a single basal, subapical or apical inoperculate discharge pore and rhizoids that arise from a subsporangial swelling, the apophysis. Rhizoids fine or coarse tapering to pointed tips. Zoospores are fully cleaved within

sporangium and are released into an evanescent vesicle in which they swarm before swimming away. Zoospores are posteriorly uniflagellate. Primary nucleus in incipient sporangium enlarges, undergoing mitosis when the sporangium is fully expanded.

Rhizoclosmatium globosum H. E. Petersen, Journ. de Botanique 17: 216, FIGS. 1, 2, 1903.
= *Phlyctochytrium powhatanensis* Roane; Mycologia 65: 535, 1973.
Mycobank MB 225931 Fig. 3

Typification: DENMARK. Séeland, from water on insect exuviae, **LECTOTYPE designated here,** MBT385827, FIG. 1 in H. E. Petersen. Journ. de Botanique 17:217, 1903. **EPITYPE designated here,** MBT385828, to support the lectotype**:** strain JEL 347h, preserved as metabolically inactive, cryopreserved and deposited in CZEUM (University of Michigan), this publication.

Description: Light tan to pink white in culture. Sporangium: hyaline, globose, subspherical, 17-22 µm (sometimes up to 35 µm); walls smooth. Apophysis: fusiform, subspherical, pyramidal and variable with rhizoids extending laterally and dichotomously branched. Rhizoids: originate laterally from apophysis but can extend also from basal portion, extensive, delicate, finely branched, and pointed at the tips. Discharge: from single inoperculate pore, apical, subapical, lateral or basal. Zoospores: elongate or ovoid 2-3 µm x 3-4 µm; contain a single large colorless lipid globule; Resting spores: 11-20 µm x 8-14 µm.

Substrate: Saprotrophic on insect exuviae, chitin, cellulose, pollen, keratin, and depleted filaments of *Aphanomyces*.

Designated Habitat of Type: Water, Denmark.

Comments: Peterson (1903) mentions FIGS. 1 and 2 but did not explicitly designate a type nor indicate whether or not the figures were based on a single gathering. Thus, FIG.1 from Petersen (1903) is here designated as the lectotype. *A culture is selected as an epitype in support of the lectotype as allowed (Turland et al., 2018).*

Sparrow (1937) reported resting spores formed sexually by rhizoidal anastomosis between contributing thalli (Sparrow 1937); but because observations were of a mixed culture, the origin of the resting spores cannot be definitively determined; germination was not observed. Although the original description (Petersen, 1903) did not describe basal zoospore discharge, Sparrow indicated basal discharge in this species (Sparrow, 1960). We did not observe basal discharge in strain JEL 347h. The empty sporangium does not collapse (Fig. 3I).

Rhizoclosmatium aurantiacum Sparrow, Proc. Amer. Phil. Soc. 78: 40, FIGS 14-17, 1937.
Mycobank MB250877
= *Rhizoclosmatium globosum* H. E. Petersen 1903, pro parte, Journ. de Botanique 17: 216.

Typification: DENMARK, Gribskov, from water on exuviae of caddisfly. **LECTOTYPE designated here,** MBT385829, Plate 2 FIG. 17 in F. K. Sparrow. Proc. Amer. Phil. Soc. 78: 40; Plate 2, not paginated, 1937.

Description: Sporangium: orange, globose, smooth surface, 27-38 µm in diam. Apophysis: broadly fusiform with rhizoids branching from lateral sides. Rhizoids: delicate, extensive, and branched. Discharge: from single inoperculate pore, basal near the apophysis. Zoospores: elliptical 2.5 x 2.0 µm; contain small orange lipid globule. Resting spores: not observed.

Substrate: Saprotrophic in water on insect exuviae, chitin, cellulose, and pollen.

Designated Habitat of Type: Denmark, water on insect exuviae.

Comments: Petersen (1903) included orange thalli among his circumscription of *R. globosum*, considering them variants due to age or other environmental factors. Sparrow (1937) recognized an orange-colored *Rhizoclosmatium* as a new species. When Sparrow (1937) described *Rhizoclosmatium aurantiacum,* he did not explicitly designate a type, but FIGS. 14-17 were indicated and mentioned. However, these illustrations were from material from two different gatherings (FIGS. 14 and 17 from Danish material and FIG. 15 from United States material). Thus from among these figures, a single illustration (FIG. 17) from a single gathering in Denmark is designated here as the lectotype of *Rhizoclosmatium aurantiacum*.

Although we have isolated orange-colored strains of *Rhizoclosmatium*, none have produced a basal discharge pore near the apophysis as Sparrow (1937) describes for *R. aurantiacum*.

Rhizoclosmatium marinum Kobayasi and M. Ookubo, Bull. Nat. Sci. Mus., Tokyo, N.S. 1, 2 (35): 68, FIG. 7, 1954.

Mycobank MB305143

Typification: JAPAN. CHIBA PREFECTURE: Anegasaki, from marine waters on *Codium fragile* **LECTOTYPE designated here,** MBT385830, FIG. 7 in Kobayasi and Ookubo. Bull. Nat. Sci. Mus., Tokyo, N.S. 1, 2 (35): 69, 1954.

Description: Sporangium: hyaline; subglobose, oval, ellipsoidal; 30-40 x 30-60 µm. Apophysis: relatively large, globose, fusiform, transversely elongated or irregular. Rhizoids: coarse, extensive, sometimes thick at the base, two to three axes branching from the base of the apophysis. Discharge: through inoperculate basal pore into vesicle where zoospores swarm before swimming away. Zoospores: ellipsoidal 7 x 5 µm. Resting spores: not observed.

Substrate: Parasitic on the marine green alga, *Codium fragile.*

Designated Habitat of Type: Anegasaki, along coastal region of Chiba Prefecture, Honshu, Japan.

Comments: Although the authors did not explicitly designate a type, they mention FIG. 7, which is designated here as the lectotype. Because of its parasitic nature and marine habitat, this is a questionable species of the genus. Isolation and culture of this species are needed to allow the molecular and morphological analyses required to determine its relationship.

Rhizoclosmatium hyalinum Karling, Sydowia 20:101, FIGS. 48-59, 1967.

Mycobank MB338322

Typification: NEW ZEALAND. Dunedin, from water in outdoor tub with *Elodea* at University of Otago. **LECTOTYPE designated here,** MBT385831**,** Plate XIX FIG. 55 in Karling. Sydowia 20: 101 Plate 19, not paginated, 1967.

Description: Sporangium: hyaline; ovoid and slightly flattened at base, 38-45 x 48-64 µm, sometimes lobose. Apophysis: irregular, elongated transversally, 8-10 x 17-23 µm, subspherical 12-18 µm; rhizoids arising from several points on periphery. Rhizoids: arise from several points on periphery of apophysis, coarse, sparingly branched, extending up to 250 µm; thick walled and appearing stiff. Discharge: basal or lateral, inoperculate; discharge pore large, up to 18 µm diam. or discharge tube 3-4 µm diam. 4-12 µm long; containing a gelatinous plug; zoospores cleaved in sporangium and released externally into a vesicle where they swarm before swimming away. Zoospores: spherical 4.6-5 µm diam.; single large lipid globule. Resting spores: smooth, thick hyaline wall, subspherical 26-30 µm diam.; ovoid 20-26 x 30-34 µm; germination not observed.

Substrate: Saprotrophic on insect exuviae and chitin.

Designated Habitat of Type: Outdoor tub with *Elodea*, Botany Department, University of Otago, Dunedin, New Zealand.

Comments: No type was explicitly designed by author (Karling, 1967) but FIGS. 48-59 were indicated and mentioned (Articles 40.3, Turland et al., 2018). Valid publication of species on or after 1 January 1958 requires indication of type (Article 40.1), which is done here with a lectotype selected as a single illustration from among FIGS. 48-59 as permitted (Articles 40.3, 40.4). In discussion of this species, Karling (1967) emphasized the large size of the apophysis in relationship to the sporangium and the conspicuous enlarging primary nucleus during development of the incipient sporangium.

Rhizoclosmatium sparsum M. J. Powell and Letcher, *sp. nov.*

Mycobank 829907 Fig. 4

Typification: UNITED STATES, Alabama, Tuscaloosa, The University of Alabama, smaller Marr's Spring pond. From an aquatic sample containing bladderwort collected by M. J. Powell and baited with chitin, strain MP 56 isolated by M. J. Powell, HOLOTYPE Fig. 4G, this publication.

Ex-Type Strain: MP 56 deposited in CZEUM (University of Michigan)

Etymology: Latin *sparsum*, spread out, referring to the rhizoids.

Description: Sporangium: hyaline; spherical, 25-40 µm diam. Apophysis: spherical, 6-8 µm diam.; broadly ellipsoidal 4-5 x 7-8µm; prolate spheroidal, 6-8 x 4-5 µm; rhizoids arising from several points on the periphery or basally. Rhizoids: thin, sparsely and widely branched, extending up to 100 µm; septum dome-shaped protruding into the sporangium. Discharge: apical or subapical inoperculate pore; zoospores cleaved in sporangium and slowly swim inside before being released externally into a vesicle, where they swarm before swimming away. Zoospores: spherical 4 µm diam., to broadly ellipsoidal 4 µm x 5 µm, contain a single lipid globule. Resting spores: not observed.

Substrate: Saprotrophic on chitin and pollen.

Designated habitat of type: Marr's Spring, The University of Alabama, Tuscaloosa, AL.

Comments: Differs from *R. globosum* by production of less dense and more widely and sparsely branched rhizoids (Fig. 4D-H) with more trunk-like-extensions from the apophysis, and more oblong to tuberous apophysis (Fig. 4G-I, K). Zoospores are spherical to broadly oval when swimming (Fig. 4A) in contrast to the more elongate zoospore of *R. globosum.*

Additional specimens examined: UNITED STATES, North Carolina, Rutherford County, Broad River at base of Bill Mountain. From aquatic sample containing plant roots covered with periphyton collected by W. H. Blackwell and baited with chitin, strain WB 266C isolated by M. J. Powell; UNITED STATES, Alabama, Tuscaloosa, Marr's Pond. From aquatic sample baited with pollen, strain MP 46 isolated by M. J. Powell.

GenBank sequences of ex-type strain MP 56: JX905524 (28S rDNA), JX905553 (ITS1-5.8S-ITS2).

Rhizoclosmatium umbonatum Letcher, Longcore, and M. J. Powell, sp. nov.

Mycobank 829908 Fig. 5

Typification: UNITED STATES, Alabama, Wheeler National Wildlife Refuge. From an aquatic sample collected 9 August 2009 by B. Swan and baited with pollen; strain MP 44 isolated by M. J. Powell, HOLOTYPE Fig. 5I, this publication.

Ex-Type Strain: MP 44 deposited in CZEUM (University of Michigan)

Etymology: Latin *umbonatum*, indicating a rounded protuberance on the developing sporangium, from germling to maturity, where the discharge pore occurs.

Description: Sporangium: light orange to tan, spherical, 45-50 µm diam. at maturity; germlings, developing sporangia, and mature sporangia have an umbo that occurs where the discharge pore will later develop. Apophysis: variable, spherical, elongate, angular, often lobed and compound; rhizoids arising from several points on the periphery. Rhizoids: coarse, densely branched, extending up to 75 µm. Discharge: apical to subapical inoperculate discharge pore containing a thick gelatinous plug; zoospores cleaved within sporangium, slowly swarm inside, and released externally into a vesicle where they swarm before swimming away. Zoospores: spherical 4 µm diam., containing a single lipid globule. Resting spores: Not observed.

Substrate: Chitin and pollen

Designated Habitat of Type: Waters edge at Wheeler National Wildlife Refuge, AL.

Comments: This new species differs from other described species of *Rhizoclosmatium* in the persistence of an unexpanded portion of the zoospore cyst throughout development of the zoosporangium. In early development, the encysted zoospore produces a germ tube (Fig. 5B). The proximal portion of the germ tube expands (Fig. 5C), becomes confluent with the basal part of the encysted zoospore, and forms the incipient sporangium (Fig. 5 D-F). The apical portion of the zoospore cyst does not expand (Figs. 5C-E), remaining on the sporangium as rounded protuberance, the umbo (Fig. 5F). The distal portion of the germ tube develops into the rhizoidal system (Figs. 5C-F) with the apophysis enlarging after the formation of the incipient sporangium (Fig. 5G). As the thallus continues developing, the umbo remains, although it diminishes in size as the sporangium expands and partially incorporates it (Fig. 5F-H, J). Because the umbo is located at only one site on the sporangium, presumably at the location of the discharge pore, it is more difficult to locate on mature sporangia (Fig. 5J) than at earlier stages (Fig. 5F, G).

Additional specimens examined: UNITED STATES, Alabama, Hale County, Oakmulgee District of Talladega National. From dragonfly wings collected in a vernal pool aquatic sample, strain WJD 185 isolated by W. J. Davis.

GenBank sequences of ex-type strain MP 44: KF257907 (28S rDNA).

Rhizoclosmatium umbonatum Letcher, Longcore, and M. J. Powell var. *sphaericum* Letcher, Longcore, and M. J. Powell, var. nov.

Mycobank 829909 Fig. 6

Typification: UNITED STATES, Maine, Hancock County, Mud Pond, aquatic sample baited with cellulose; strain JEL 128 isolated by J. E. Longcore, HOLOTYPE Fig. 6J, this publication.

Ex-Type Strain: JEL 128 deposited in CZEUM (University of Michigan)

Etymology: Latin *sphaericum*, to indicate the spherical shape of the apophysis, which is distinguishable from the irregular, often compound apophysis of *R. umbonatum*.

Description: Sporangium: dark orange, suboblate to spherical during development, spherical at maturity, 30-35 µm diam.; germlings, developing sporangia, and mature sporangia have an umbo that occurs where the discharge pore will develop. Apophysis: predominantly spherical, 8-10 µm diam., rarely multi-lobed; rhizoids arising from several points on the periphery including basally. Rhizoids: dense, thin, widely branched, extending up to 80 µm. Discharge: apical to subapical inoperculate discharge pore containing a thick gelatinous plug; zoospores cleaved in sporangium, slowly swarm inside, released externally into a vesicle where they swarm before swimming away. Zoospores: spherical 4 µm diam., to slightly oval, 5 µm long. Resting spores: not observed.

Substrate: Cellulose and chitin

Designated Habitat of Type: Acidic lake, Mud Pond, Hancock County, ME.

Comments: As in *R. umbonatum,* a vestige of the encysted zoospore persists on the sporangium (Fig. 6E, F). The predominantly spherical apophysis of *R. umbonatum* var. *sphaericum* (Fig. 6H, J) makes it distinct from *R. umbonatum* with a predominantly multi-lobed apophysis (Fig. 5I). Apophysis begins to form after the incipient sporangium has started to expand (Fig. 6D versus 6E).

Additional specimens examined: UNITED STATES, Maine, from aquatic sample collected in 2006 from Perch Pond, Old Town, Penobscot County and baited with chitin, strain JEL 516 isolated by J. E. Longcore. UNITED STATES, Maine, from aquatic sample collected 1 June 2013 from Perch Pond, Old Town, Penobscot County and baited with chitin, strain JEL 796 isolated by J. E. Longcore.

GenBank sequences ex-type strain JEL 128: MK328908 (28S rDNA).

Rhizoclosmatium persicum Letcher, Longcore, and M. J. Powell, sp. nov.

Mycobank 829910 Fig. 7

Typification: UNITED STATES, Alabama, Tuscaloosa County. From an aquatic sample collected at the Lake Nicol dam spillway and baited with cellulose; strain MP 067 isolated by M. J. Powell, HOLOTYPE Fig. 7C, this publication.

Ex-Type Strain: MP 067 deposited in CZEUM (University of Michigan)

Etymology: Latin *persicum*, referring to bright orange flesh of the fruit of the apricot tree, *Prunus armeniaca*.

Description: Sporangium: orange; spherical, typically 25-45 µm diam., sometimes 55 µm diam.; primary nucleus continues to enlarge and divides after sporangium expansion is complete. Germling: encysted zoospore produces a long germ tube with few lateral branches; tip of germ tube bifurcates branches; apophysis produced later. Apophysis: spherical, subspherical, oval, angular pyramidal, or campanulate; most rhizoids extend from the sides of the apophysis. Rhizoids: primary axes stout; rhizoids moderately dense, finely branched; dome-shaped septum delimits rhizoid system from sporangium. Discharge: subapical to lateral inoperculate discharge pore; zoospores cleaved in sporangium, swarm in sporangium prior to discharge, released externally into a vesicle where they continue to swarm before swimming away. Zoospores: ellipsoidal 4 µm diam. x 5 µm length, single lipid globule. Resting spores: not observed.

Substrate: Cellulose and chitin

Designated Habitat of Type: Dam spillway, Lake Nicol, Tuscaloosa County, AL.

Additional specimens examined: UNITED STATES, Alabama, Tuscaloosa County, Coker, Lake Lurleen. From bank surface water sample baited 27 February 2008 with cellulose, strain EL 102 isolated by E. Lefèvre. UNITED STATES, Alabama, Hale County, Oakmulgee District of Talladega National. From *Daphnia* collected in a vernal pool aquatic sample, strain WJD 187 isolated by W. J. Davis; UNITED STATES, North Carolina, Rutherford County, Lake Lure. From aquatic sample containing green algae collected by W. H. Blackwell and baited with chitin, strain MP 14 isolated by M. J. Powell; UNITED STATES, North Carolina, Rutherford County, Lake Lure. From aquatic sample containing periphyton on aquatic plant stem collected by W. H. Blackwell and baited with chitin, strain MP 15 isolated by M. J. Powell.

GenBank sequences ex-type strain MP 067: KC691343 (28S rDNA).

Comments: The primary nucleus continues to enlarge as the sporangium expands (Fig. 7B). In the later production of an apophysis on the germ tube (Fig. 7A versus Fig. 8B) and more globose to pyramidal-shaped apophysis (Fig. 7B, C, D, F versus Fig. 8C, D, H), *R. persicum* differs from *R. pessaminum*. Zoospore discharge from a lateral pore is shown in Fig. 7D-E.

Rhizoclosmatium pessaminum Letcher, Longcore, and M. J. Powell, sp. nov.

Mycobank 829911 Fig. 8

Typification: UNITED STATES, Maine, from aquatic sample collected from Perch Pond, Old Town, Penobscot County in 2014 and baited with chitin, strain JEL 823 isolated by J. E. Longcore, HOLOTYPE Fig. 8D, this publication.

Ex-Type Strain: JEL 823 deposited in CZEUM (University of Michigan)

Etymology: 'pessamin', from the Powhatan language, an Algonquin language of the eastern United States, referring to the orange fruit of the American persimmon, *Diospyros virginiana*.

Description: Sporangium: orange; spherical, 30-50 µm diam. Apophysis: prolate to transversally elongate, 8-10 x 18-20 µm; rhizoids arising from several points on the periphery. Rhizoids: moderately dense, finely branched, extending up to 120 µm. Discharge: subapical to lateral inoperculate discharge pore containing a thin gelatinous plug; zoospores cleaved in sporangium, swarm in sporangium prior to discharge, released externally into a vesicle where they continue to swarm before swimming away. Zoospores: broadly ellipsoidal 4 µm diam. x 5 µm length, containing one to two lipid globules. Resting spores: not observed.

Substrate: Chitin

Designated Habitat of Type: Edge of Lake, Fen, Perch Pond, ME.

Additional specimens examined: UNITED STATES, Maine, from aquatic sample collected from Perch Pond, Old Town, Penobscot County in 2015 and baited with chitin, strain JEL 849 isolated by J. E. Longcore.

GenBank sequences ex-type strain JEL 823: MK328911 (28S rDNA).

Comments: The common presence of two lipid globules in a zoospore (Fig. 8A) is unlike the other described species. The apophysis forms early in germling development (Fig. 8B), and varies in shape from transversely elongate (Fig. 8C, D), oval (Fig. 8G) to prolate (Fig. 8H). A domed-shaped septum delimits the rhizoids from the sporangium (Fig. 8G, H). Zoospore discharge is through a subapical to lateral pore (Fig. 8E-G). This chytrid produced sporangia of two distinct sizes, one being 30–35 µm diam., the other being 50–60 µm diam. (Fig. 8I). The smaller sporangia are usually the sporangia that complete zoosporogenesis (Fig. 8I). The larger sporangia most often abort before zoosporogenesis; partially collapsed large sporangia often retain granular material, although a very few large sporangia had cleaved contents. It is known that *R. globosum* becomes hypertrophied when infected with the endoparasite *Rozella rhizoclosmatii* (Letcher et al., 2017), but we observed no indication of infection in these large thalli.

KEY TO SPECIES OF *RHIZOCLOSMATIUM*

1. Parasitic on marine green alga . . . . . *R. marinum*
1. Saprotrophic on chitin, pollen, cellulose or keratin 2
2. Hyaline, rose-white to light tan in culture 3

2. Light orange to dark orange in culture 5
3. Sporangia ovoid and sometimes lobed; rhizoids thick-walled and coarse . . . . . *R. hyalinum*
3. Sporangia spherical to subspherical; rhizoids thin-walled and delicate 4
4. Apophysis transversely elongate . . . . . .*R. globosum*
4. Apophysis spherical, ovoid to longitudinally tuber-shaped . . . . . *R. sparsum*
5. Germling with a prominent apical umbo 6
5. Germling lacking a prominent apical umbo 7
6. Apophysis predominantly multilobed at maturity . . . . . .*R. umbonatum*
6. Apophysis predominantly spherical to ovate at maturity . . . . . .*R. umbonatum* var. *sphaericum*
7. Zoospore discharge pore typically basal . . . . . *R. aurantiacum*
7. Zoospore discharge pore typically other than basal 8
8. Apophysis subspherical, oval to pyramidal with stout rhizoidal axes . . . . . .*R. persicum*
8. Apophysis transversely elongate to prolate, rhizoids dense . . . . . .*R. pessaminum*

DISCUSSION

Of the now eight species in *Rhizoclosmatium*, we were able to compare the morphology of five in pure culture and provide 28S rDNA sequence information to aid in their identification. Our study of strains grown in pure culture confirms the common characteristics of *Rhizoclosmatium.* In all species the primary nucleus enlarged in the incipient sporangium and divided only after the sporangium reached full size, as reported in several other chytrid species (Antikajian, 1949). A rhizoidal apophysis and domed-shaped septum separating the sporangium and apophysis were produced. As typical for members of the Chytridiales (Letcher and Powell, 2014), rhizoids were finely branched and tapered toward the tips. Early reports of *Rhizoclosmatium* were based on growth on natural substrates, and Sparrow (1960) indicated that basal discharge was common (Sparrow, 1960). With the strains we observed in culture, the single inoperculate discharge pore varied in position from apical, subapical, to lateral, but we did not observe basal discharge. Consistent with prior observations, zoospore discharge was vesicular.

The major morphological differences between species delimited in this study are complexity of rhizoidal system, shape of apophysis, and sporangial size and color in culture. Because of the plasticity of thallus morphology that chytrids typically exhibit (Powell and Koch, 1977), variability in these features is common. Variation in apophysis and rhizoidal structure ascribed to some strains of *R. globosum* in earlier studies, however, may reflect the possibility that other researchers have also observed strains that are new species of *Rhizoclosmatium*. For example, Johnson (1973) noted that strains from Iceland he considered as *R. globosum* often had apophyses that were large, irregular and sometimes cylindrical. We have also discovered that a chytrid strain described as *Phlyctochytrium powhatanensis* ATCC 22918 (Roane, 1973) is actually a strain of *R. globosum* (Fig. 1), most likely earlier confused because *Phlyctochytrium* produces an inoperculate discharge pore and apophysate thallus. Interestingly the strain was isolated from a chitin containing organism, a rotifer (Roane, 1973), a substrate on which *Rhizoclosmatium* can be expected. Consistent with the molecular results, Barr (1980, pg. 2388) earlier reported this strain had a Group I zoospore.

Morphological and molecular phylogenetic analyses support the circumscription of four new species and one new variety in this genus. *Rhizoclosmatium sparsum* produces hyaline to light tan thalli, as in *R. globosum. Rhizoclosmatium umbonatum, R. persicum,* and *R. pessaminum* produce light orange to bright

orange thalli, as in *R. aurantiacum.* When Petersen (1903) originally described *Rhizoclosmatium globosum*, he included both white/rose-colored and bright-orange-colored forms. Both forms were commonly collected from the same site and could occur together on the same piece of chitin. Sparrow (1937) recognized that strains with orange thalli and orange lipid globules in zoospores were distinct from *R. globosum*, and described *R. aurantiacum* as a new species. Unlike our orange-colored strains, zoospore discharge in *R. aurantiacum* was described as basal, with the pore near the apophysis (Sparrow, 1937).

Barr and Hartmann (1976) first described the zoospore ultrastructure of *Rhizoclosmatium globosum* (strain BR 34–ATCC 22197), and Barr (1980) designated it as a group I zoospore among the *Chytridiales* types of zoospores. More recent ultrastructural and molecular phylogenetic studies have used differences in zoospore ultrastructure to delineate the family Chytriomycetaceae from the family Chytridiaceae in the Chytridiales (Letcher and Powell, 2014; Vélez et al., 2011). The *Rhizoclosmatium* strains we studied had zoospores with the features of a group I zoospore as Barr (1980) described and Letcher and Powell (2014) further defined. The only difference among strains was the position of the nucleus within the ribosomal aggregation. Among the orange pigmented strains, the nucleus was partially inserted into the ribosomal aggregation in *R. persicum* (strains MP 67, WJD 187) and *R. umbonatum* (strains MP 44, WJD 185); but among the more hyaline strains, such as *R. globosum* (strain WJD 111), the nucleus, although still partially inserted into the ribosomal aggregation, was not as deeply inserted. These results are consistent with Letcher and Powell's (2014) hypothesis based on analysis of character state evolution. They proposed that nuclear location in the zoospore is a neutral character in natural selection. In the last common ancestor of Chytridiales, the nucleus was located within the ribosomal aggregation; nuclear progressive movement toward the surface of the ribosomal aggregation and then to the periphery occurred in independent linages.

Chytrids are recognized as important degraders of refractory material including cellulose, chitin, keratin and pollen (Goldstein, 1960; Tribe, 1957). Recent ecological studies demonstrate that chytrids have a role in the 'Mycoloop" in transforming materials such as pollen, which many zooplankton cannot use, into a form which they can use, nutritionally rich chytrid zoospores (Kagami et al., 2007b, 2014, 2017). Species in the genus *Rhizoclosmatium* are considered chitin degraders (Sparrow, 1960), but based on our sampled strains, are also able to break down other substrates including pollen as well as keratin and cellulose-containing substrates. Descriptions of sites for collection of *Rhizoclosmatium* species often indicate an association with aquatic vegetation. Thus, *Rhizoclosmatium* species, which are common in aquatic habitats, may have a significant role in nutrient cycling and productivity of aquatic habitats. The complete genome of *R. globosum* (JEL 800) is available from the US DOE Joint Genome Institute, making it possible to determine its enzymatic capabilities (Mondo et al., 2017). With the widespread occurrence of *R. globosum*, its ease of culture, its generation time of ~20 hrs. at 23° C, and the availability of its genetic information, *R. globosum* can be a valuable model organism. The importance of the genus *Rhizoclosmatium* in nutrient cycling and sustainability in aquatic habitats is an important avenue to investigate further.

CONCLUSIONS

This study has shown that species diversity within *Rhizoclosmatium* is greater than earlier realized. Because chytrids exhibit thallus plasticity, identification of species based on morphology alone is problematic. Consequently, molecular phylogenetics inform our understanding of species distinctions within this genus. Significantly, zoospore ultrastructure is conserved within the genus. Surveys of chytrid biodiversity have demonstrated that a few chytrid species are essentially ubiquitous and many are scarce to rare (Davis et al., 2013; Letcher and Powell, 2001; Letcher et al., 2004, 2006, 2008a, 2008b). We have previously shown that for chytrid taxa that are common, local sampling tends to reflect global diversity (Davis et al., 2013; Letcher et al., 2008b). The geographic sampling in this study was somewhat limited,

predominantly southeastern and northeastern United States, yet species diversity was revealed. From this sampling we found that *R. globosum* was a common taxon and the newly described species were rarer. These results suggest that additional sampling could reveal greater diversity in this genus.

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LITERATURE CITED

Antikajian, G. 1949. A developmental, morphological, and cytological study of *Asterophlyctis* with special reference to its sexuality, taxonomy, and relationships. Am. J. Bot. 36: 245-262.

Barr, D. J. S. 1980. An outline for the reclassification of the Chytridiales, and for a new order, the Spizellomycetales. Canad. J. Bot. 58: 2380-2394.

Barr, D. J. S. and V. E. Hartmann. 1976. Zoospore ultrastructure of three *Chytridium* species and *Rhizoclosmatium globosum*. Canad. J. Bot. 54: 2000-2013.

Davis, W. J., P. M. Letcher and M. J. Powell. 2013. Chytrid diversity of Tuscaloosa County, Alabama. Southeast. Nat. 12: 666-683.

Davis, W. J., J. Antonetti, P. M. Letcher and M. J. Powell. 2016. Phylogenetic diversity of Chytridiomycetes in a temporary forest pond surveyed using culture-based methods. Southeast. Nat. 15: 534-548.

Davis, W. J., K. T. Picard, J. Antonetti, J. Edmonds, J. Fults, P. M. Letcher and M. J. Powell. 2018. Inventory of chytrid diversity in two temporary forest ponds using a multiphasic approach. Mycologia 110: 811-821, DOI: 10.1080/00275514.2018.1510725.

Goldstein, S. 1960. Degradation of pollen by phycomycetes. Ecology 41: 543-545.

Gooday, G. W. 1990. The ecology of chitin degradation. *in* Advances in Microbial Ecology volume 11. K.C. Marshall, ed., Plenum Press, New York. p. 387–430.

Hall, T. A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucleic Acids Symp. Ser. (Oxf.) 41: 95-98.

James, T. Y., P. M. Letcher, J. E. Longcore, S. E. Mozley-Standridge, D. Porter, M. J. Powell, G. W. Griffith and R. Vilgalys. 2006. A molecular phylogeny of the flagellated fungi (Chytridiomycota) and description of a new phylum (Blastocladiomycota). Mycologia 98: 860-871, doi:10:10.3852/mycologia. 98.6.860.

Johnson, T. W. 1973. Aquatic fungi of Iceland: Uniflagellate species. Acta Nat. Island. 22: 1-38.

Kagami, M., A. de Bruin, B. W. Ibelings and E. Van Donk. 2007a. Parasitic chytrids: their effects on phytoplankton communities and food-web dynamics. Hydrobiologia 578: 113-129, doi: 10.1007/s10750-006-0438z.

Kagami, M., E. von Elert, B. W. Ibelings, A. de Bruin and E. Van Donk. 2007b. The parasitic chytrid, *Zygorhizidium*, facilitates the growth of the cladoceran zooplankter, *Daphnia*, in cultures of the inedible alga, *Asterionella*. Proc. R. Soc. B 274: 1561-1566, doi: 10.1098/rspb.2007.0425.

Kagami, M., N. R. Helmsing and E. Van Donk. 2011. Parasitic chytrids could promote copepod survival by mediating material transfer from inedible diatoms. Hydrobiologia 659: 49-54, doi: 10.1007/s10750-010-274-z.

Kagami, M., T. Miki and G. Takimoto. 2014. Mycoloop: chytrids in aquatic food webs. Front. Microbiol. 5: 166, doi: 10.3389/fmicb. 2014.00166.

Kagami, M., Y. Motoki, H. Masclaux and A. Bec. 2017. Carbon and nutrients of indigestible pollen are transferred to zooplankton by chytrid fungi. Freshwater Biol. 62: 954-964.

Karling, J. S. 1967. Some zoosporic fungi of New Zealand. V. Species of *Asterophlyctis, Obelidium, Rhizoclosmatium, Siphonaria* and *Rhizophlyctis*. Sydowia 20: 96-108.

Keyhani, N. O. and S. Roseman. 1999. Physiological aspects of chitin catabolism in marine bacteria. Biochim. Biophys. Acta 1473: 108-122.

Kobayasi, Y. and M. Ookubo. 1954. Studies on the marine phycomycetes II. Bull. Nat. Sci. Mus., Tokyo, N.S. 1, 2 (35): 62-71.

Lefèvre, E., P. M. Letcher and M. J. Powell. 2012. Temporal variation of the small eukaryotic community in two freshwater lakes: emphasis on zoosporic fungi. Aquat. Microb. Ecol. 67: 91-105.

Letcher, P. M. and M. J. Powell. 2001. Distribution of zoosporic fungi in forest soils of the Blue Ridge and Appalachian Mountains of Virginia. Mycologia 93:1029–1041, doi:10.2307/3761665

Letcher, P.M. and M. J. Powell. 2014. Hypothesized evolutionary trends in zoospore ultrastructural characters in Chytridiales (Chytridiomycota). Mycologia 106: 379-396, doi:10.3852/13-219

Letcher, P. M., M. J. Powell, J. G. Chambers and W. E. Holznagel. 2004. Phylogenetic relationships among *Rhizophydium* isolates from North America and Australia. Mycologia 96: 1339-1351, doi:10.2307/3762150

Letcher, P. M., M. J. Powell, P. F. Churchill and J. G. Chambers. 2006. Ultrastructural and phylogenetic delineation of a new order, the Rhizophydiales. Mycol. Res. 110: 898-915, doi:10.1016/j.mycres. 2006.06.011

Letcher, P. M., M. J. Powell, D. J. S. Barr, P. F. Churchill, W. S. Wakefield, and K. T. Picard. 2008a. Rhizophlyctidales-a new order in Chytridiomycota. Mycol. Res. 112: 1031-1048, doi:10.1016/ j.mycres.2008.03.007

Letcher, P. M., C. G. Vélez, M. E. Barrantes, M. J. Powell, P. F. Churchill and W. S. Wakefield. 2008b. Ultrastructural and molecular analyses of Rhizophydiales (Chytridiomycota) isolates from North America and Argentina. Mycol. Res. 112: 759-782.

Letcher, P. M., J. E. Longcore, C. A. Quandt, D. S. Leite, T. Y. James and M. J. Powell. 2017. Morphological, molecular, and ultrastructural characterization of *Rozella rhizoclosmatii*, a new species in Cryptomycota. Fungal Biol. 121: 1-10.

Mondo, S. J., R. O. Dannebaum, R. C. Kuo, K. B. Louie, et al. 2017. Widespread adenine N6-methylation of active genes in fungi. Nature Genet. 49: 964-968.

Otterholt, E. and C. Charnock. 2011. Identification and phylogeny of the small eukaryote population of raw and drinking waters. Water Res. 45: 2527-2538.

Petersen, H. E. 1903. Note sur les Phycomycètes observés dans les téguments vides des nymphes de Phryganées avec description de trois espèces nouvelles de Chytridinèes. Journ. de Botanique 17: 214-222.

Posada. D. and K. A. Crandall. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14:817–818, doi:10.1093/bioinformatics/14.9.817

Powell, M. J. and W. J. Koch. 1977. Morphological variations in a new species of *Entophlyctis*. II. Influence of growth conditions on morphology. Canad. J. Bot. 55: 1686-1695.

Powell, M. J., P. M. Letcher and J. E. Longcore. 2013. *Pseudorhizidium* is a new genus with distinct zoospore ultrastructure in the order Chytridiales. Mycologia 105: 496-507, doi:10.3852/12-269

Rasconi, S., B. Grami, N. Niquil, M. Jobard and T. Sime-Ngando. 2014. Parasitic chytrids sustain zooplankton growth during inedible algal bloom. Front. Microbiol. 5:229, doi: 10.3389/fmicb. 2014.00229

Rehner, S. A. and G. J. Samuels. 1994. Taxonomy and phylogeny of *Gliocladium* analysed from nuclear large subunit ribosomal DNA sequences. Mycol. Res. 98: 625-634, doi:10.1016/S0953–7562 (09)80409–7

Roane, M. K. 1973. Two new chytrids from the Appalachian highlands. Mycologia 65: 531-538.

Sikes, D. S. and P. O. Lewis. 2001. PAUPRat: PAUP* implementation of the parsimony ratchet. Version Beta Software. Version 1. Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, CT. Distributed by the authors.

Sparrow, F. K. 1937. Some chytridiaceous inhabitants of submerged insect exuviae. Proc. Amer. Philos. Soc. 78: 23-53.

Sparrow, F. K. 1960. Aquatic Phycomycetes. Univ. of Michigan Press, Ann Arbor. xxvi + 1187 pp.

Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin and D. G. Higgins. 1997. The Clustal X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25: 4876-4882.

Tribe, H. T. 1957. Ecology of the micro-organisms in soils as observed during their development upon buried cellulose film. Microb. Ecol. 8: 287-298.

Turland, N. G. et al. 2018. International code of nomenclature for algae, fungi and plants (Shenzhen Code). Regnum Vegetabile 159.XXXVIII, 254 p.

Vélez, C. G., P. M. Letcher, S. Schultz, M. J. Powell and P. F. Churchill. 2011. Molecular phylogenetic and zoospore ultrastructural analyses of *Chytridium olla* establish the limits of a monophyletic Chytridiales. Mycologia 103: 118-130, doi:10.3852/10-001

Vilgalys, R. and M. Hester. 1990. Rapid identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. J. Bacteriol. 172: 4238-4246.

White, T. J., T. D. Bruns, S. B. Lee and J. W. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *in* PCR Protocols: A Guide to Methods and Applications. Innis, M. A., D. H. Gelfand, J. J. Sninsky, and T. J. White, eds., Academic Press, New York. pp. 315-322.

Zwikl, D. J. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under maximum likelihood criterion [doctoral dissertation]. Austin: Univ. Texas. 115 p.

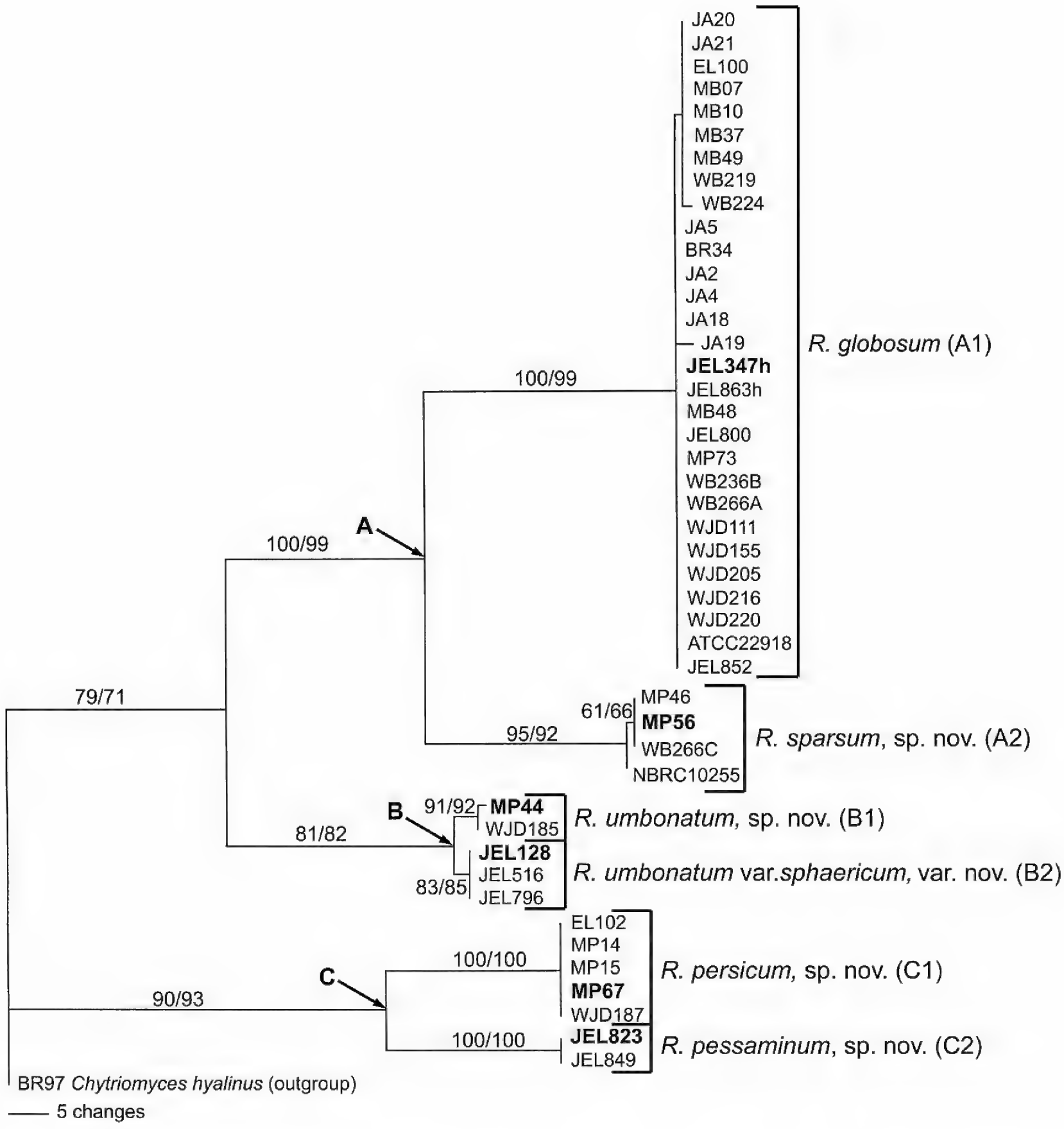

Figure 1. Molecular phylogenetic assessment. Phylogram inferred from strict consensus, maximum parsimony analysis of 45 strains of *Rhizoclosmatium* using 28S rDNA. Numbers at nodes are bootstrap support values (maximum likelihood/maximum parsimony). Three lineages are resolved (A, B, C), each with two sub-clades (A1, A2, B1, B2, C1, C2).

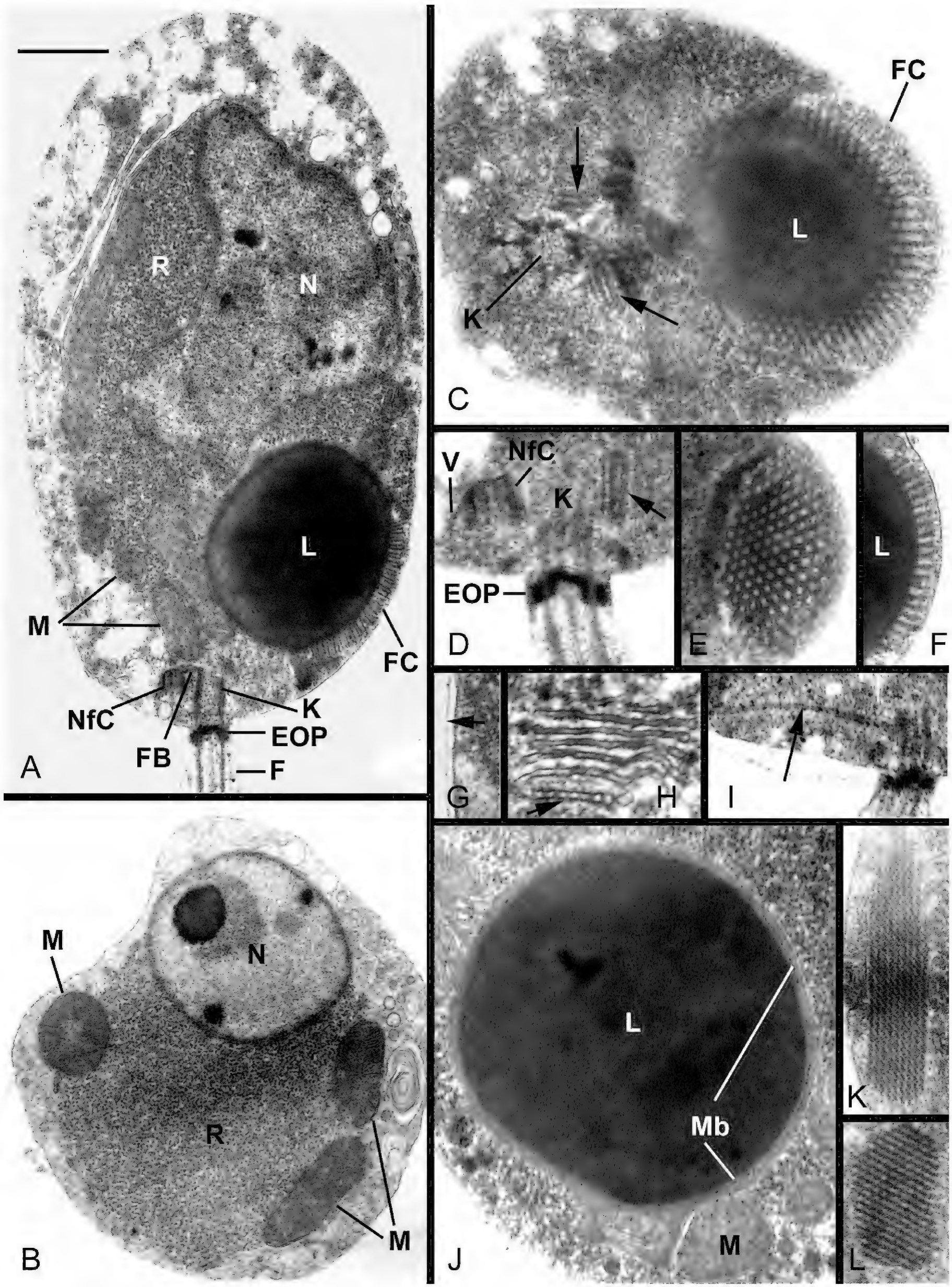

Figure 2. See caption on following page.

Figure 2. Ultrastructure of the *Rhizoclosmatium* species zoospore. A, C, E–H. *R. persicum* strain MP 67; B, K, L. *R. umbonatum* strain WJD 185; D, I. *R. globosum* strain WJD 111; J. *R. globosum* strain ATCC 22918. (A) Longitudinal section with aggregated ribosomes, a nucleus partially inserted into the ribosomal aggregation, a fenestrated MLC cisterna appressed to a single lipid globule, multiple mitochondrial profiles outside the ribosomal aggregation, a non-flagellated centriole adjacent to the kinetosome, the two connected by a fibrillar bridge, and an electron-opaque plug in the base of the flagellum. (B) TS with the nucleus partially inserted in the ribosomal aggregation, and multiple mitochondrial profiles outside the aggregation. (C) TS through the kinetosome, illustrating the KAS as a set of plates (arrows), and an oblique section through the fenestrated MLC cisterna. (D) LS illustrating a veil adjacent to the non-flagellated centriole and the KAS adjacent to the kinetosome (arrow). (E) Face view and (F) LS through fenestrated MLC cisterna. (G) Cell coat (arrow) adjacent to plasma membrane. (H) Golgi apparatus; notice partitioned cisterna (arrow). (I) LS illustrating microtubular root (arrow) extending from kinetosome toward fenestrated MLC cisterna. (J) Microbody appressed to the lipid globule. (K) LS through paracrystalline inclusion. (L) TS through paracrystalline inclusion. Scale bar = 1.0 μm (A, B, C, E, F), 0.5 μm (I), 0.4 μm (J, K, L), 0.3 μm (H), 0.25 μm (D, G). Abbreviations: EOP, electron-opaque flagellar plug; F, flagellum; FB, fibrillar bridge; FC, fenestrated MLC cisterna; K, kinetosome; KAS, kinetosome-associated structure; L, lipid globule; LS, longitudinal section; M, mitochondrion; MB, microbody; MLC, microbody-lipid globule complex; N, nucleus; NfC, non-flagellated centriole; R, ribosomal aggregation; TS transverse section; V, veil.

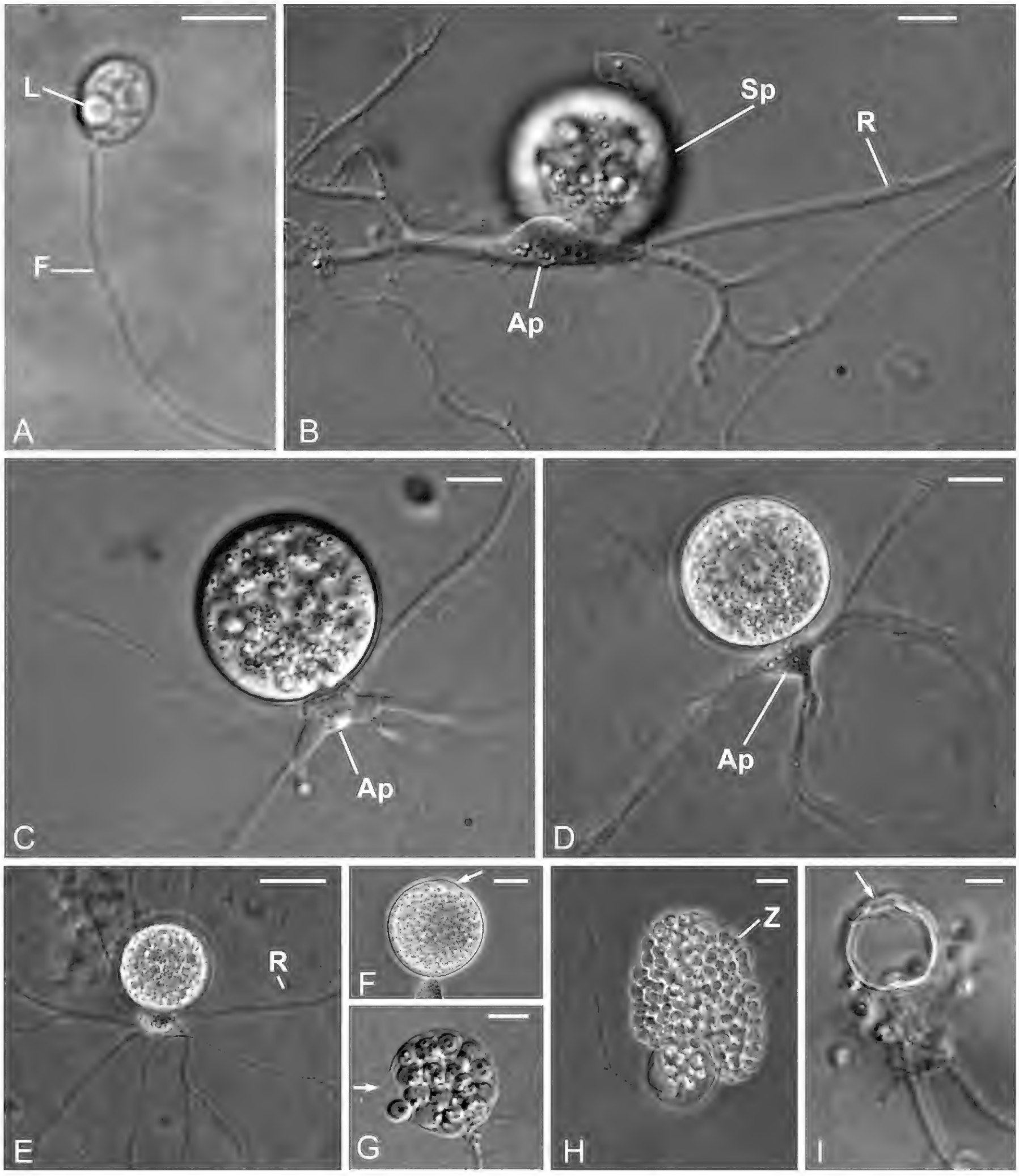

Figure 3. Thallus morphology of *R. globosum*, strain JEL 347h. (A) Motile zoospore with a single lipid globule and posterior flagellum. (B) Developing sporangium with subsporangial apophysis and rhizoids originating from multiple points on the apophysis. (C–E) Developing sporangia, the subsporangial apophyses having variable morphology. (F) Maturing sporangium with a hyaline plug located at the apical discharge pore (arrow). (G) Mature sporangium with a lateral discharge pore (arrow). (H) Vesicular discharge of zoospores. (I) Empty sporangium with apical discharge pore (arrow). Scale bar = 20 µm (E), 10 µm (B, C, D, F, G, H, I), 5 µm (A). Abbreviations: Ap, apophysis; F, flagellum; L, lipid globule; R, rhizoid; Sp, sporangium; Z, zoospore.

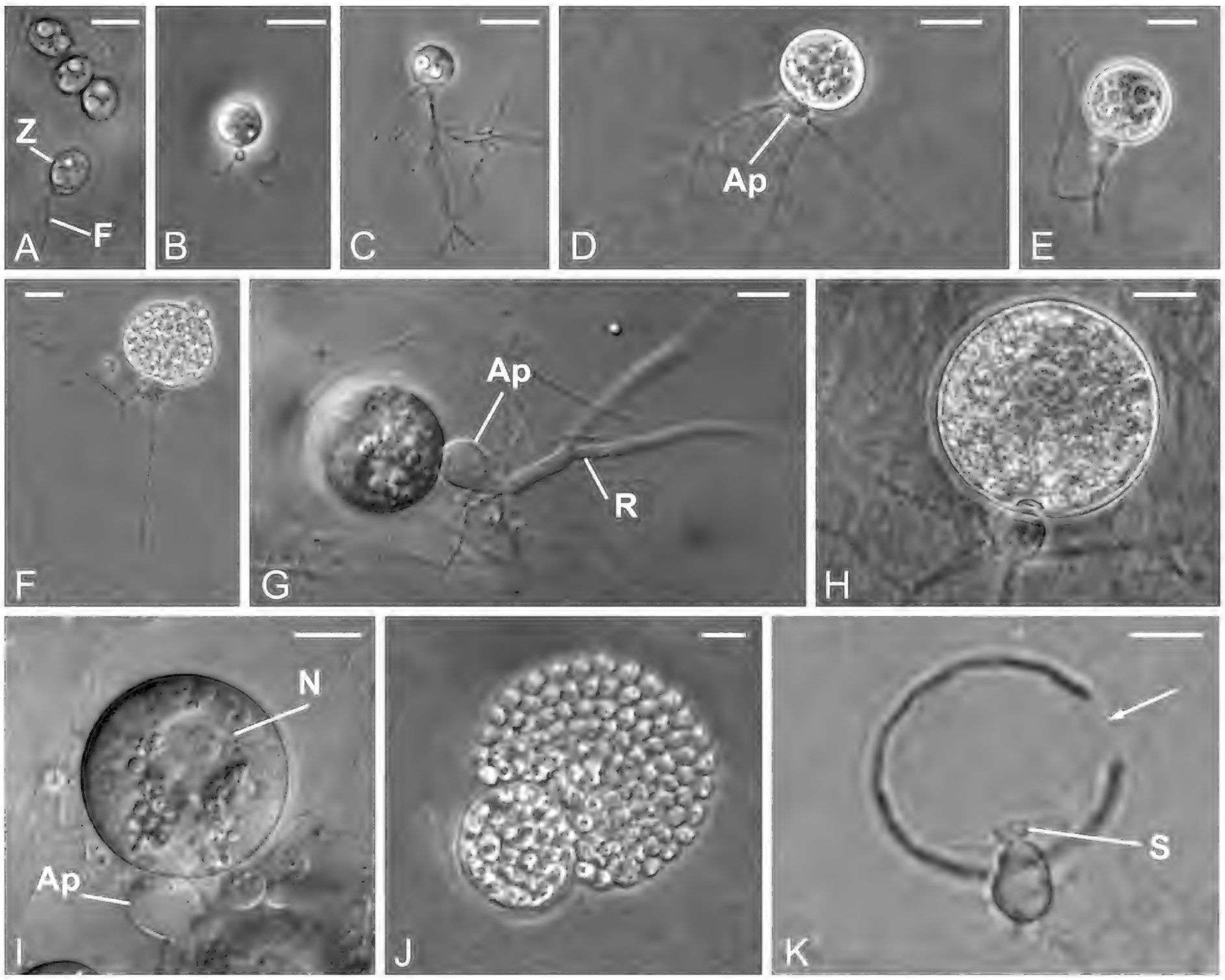

Figure. 4. Thallus morphology of *R. sparsum* sp. nov., strain MP 56. (A) Broadly oval zoospores. (B) Young sporangium with rhizoidal system extending from small, round apophysis. (C) Germling with long rhizoid. (D) Developing thallus with elongate apophysis. (E) Developing thallus with tuberous apophysis and basal, trunk-like, sparely branched rhizoid system. (F) Mature thallus with apical discharge pore; sporangium with cleaved zoospores; rhizoidal system finely branched with widely spaced rhizoids arising from base of spherical apophysis. (G) Developing thallus with tuberous apophysis and basal, trunk-like rhizoidal axis. (H) Mature, spherical sporangium; widely spaced rhizoidal axes emanating from total surface of subspherical apophysis. (I) Primary nucleus in sporangium is enlarged; apophysis subspherical. (J) Vesicular discharge of zoospores. (K) Empty sporangium with lateral discharge pore (arrow); dome-shaped septum extending from tuberous apophysis. Scale bar =10 μm (B-K), 5 μm (A). Abbreviations: Ap, apophysis; F, flagellum; N, large primary nucleus; R, rhizoid; S, septum; Z, zoospore.

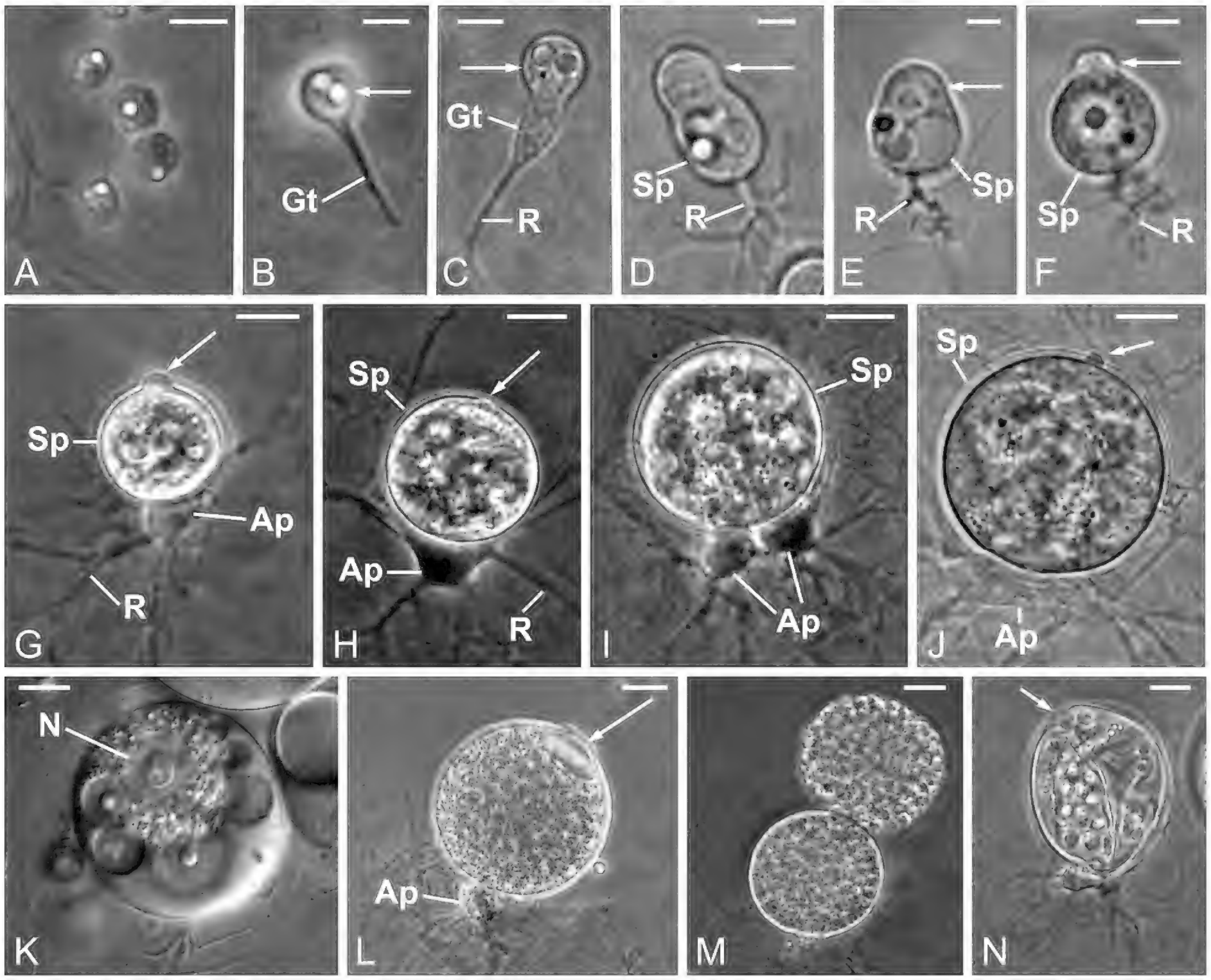

Figure 5. Thallus morphology of *R. umbonatum* sp. nov., strain MP 44. (A) Zoospores with single lipid globule. (B) Germling; encysted zoospore (arrow) with germ tube. (C) Germling; proximal portion of germ tube swells and becomes confluent with a portion of the encysted zoospore (arrow); distal portion of germ tube becomes rhizoid. (D, E, F) Early thallus development; apical portion of encysted zoospore not totally incorporated into sporangium and remains as an umbo (arrow); sporangium and rhizoid develop; apophysis not enlarged yet. (G) Immature thallus with spherical sporangium bearing an apical umbo (arrow); apophysis enlarged. (H) Developing thallus; rhizoids originate from multiple points on the apophysis; remnant of umbo (arrow). (I) Developing sporangium with multi-lobed apophysis. (J) Maturing sporangium with apophysis and remnant of umbo (arrow). (K) Maturing sporangium with large primary nucleus. (L) Mature sporangium with thick hyaline plug in the apical discharge pore (arrow); one lobe of apophysis visible. (M) Vesicular zoospore discharge. (N) Almost empty sporangium; subapical discharge pore (arrow). Scale bar = 5 μm (A–F), 10 μm (G–N). Abbreviations: Ap, apophysis; Gt, germ tube; N, large primary nucleus; R, rhizoid; Sp, sporangium.

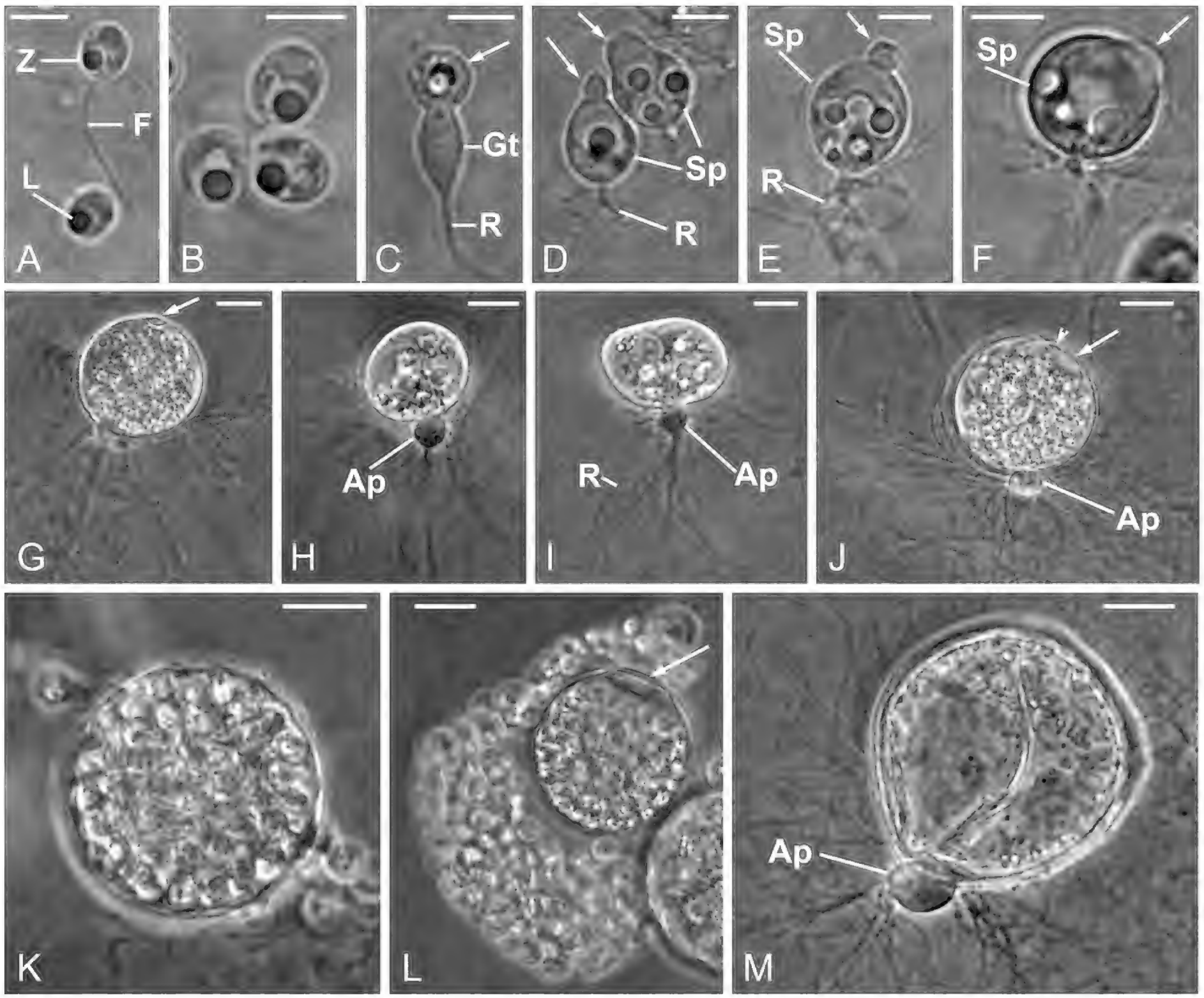

Figure 6. Thallus morphology of *R. umbonatum* var. *sphaericum*, var. nov., strain JEL 128. (A) Motile zoospores each with a single lipid globule and posterior flagellum. (B) Encysting zoospores each containing a single lipid globule. (C) Germling; encysted zoospore (arrow) with germ tube; proximal portion of germ tube expands and the distal portion develops the rhizoidal axis. (D) Two developing thalli; encysted zoospore (arrow) partially confluent with expanded portion of germ tube becoming the sporangium; apical portion of encysted zoospore only partially expanded, remaining as an apical umbo (arrow); tip of germ tube developing rhizoids. (E) Immature sporangium with an apical umbo (arrow). (F) Developing sporangium with a subapical umbo (arrow). (G) Maturing sporangium with remnant of apical umbo (arrow). (H) Maturing, subglobose sporangium with spherical apophysis. (I) Maturing, suboblate sporangium with spherical apophysis. (J) Maturing, spherical sporangium with spherical apophysis and subapical hyaline plug (arrow) at site of discharge pore; remnant of apical umbo is visible (arrowhead). (K) Mature sporangium with cleaved zoospores. (L) Vesicular discharge of zoospores; arrow indicates discharge pore. (M) Empty sporangium with spherical apophysis. Scale bar = 5 μm (A–E), 10 μm (F–M). Abbreviations: Ap, apophysis; F, flagellum; Gt, germ tube; L, lipid globule; R, rhizoid; Sp, sporangium; Z, zoospore.

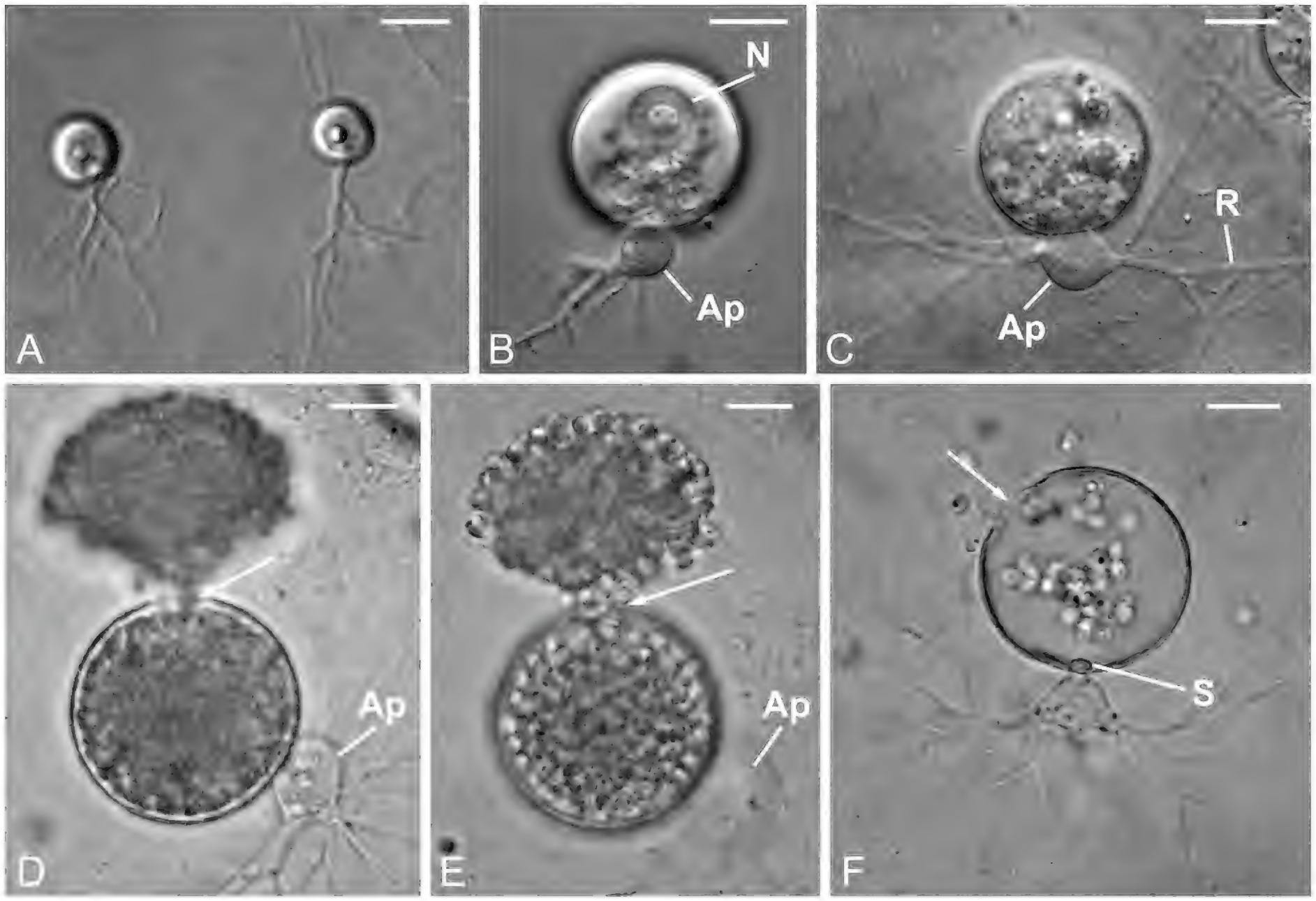

Figure 7. Thallus morphology of *R. persicum* sp. nov., strain MP 67. (A) Two germlings early rhizoidal development. (B) Developing thallus with large primary nucleus in sporangium; subglobose apophysis. (C) Maturing thallus; spherical sporangium with ovate apophysis; stout rhizoidal axes emanating from sides of apophysis. (D-E) Two focal levels through discharging sporangium showing location of angular pyramidal apophysis and lateral position of discharge pore (arrow); zoospores released from sporangium as a mass before swimming away. (F) Thallus after zoospore discharge revealing subapical discharge pore (arrow), dome-shaped septum between the sporangium and apophysis, and fine rhizoids extending from the sides of the campanulate-shaped apophysis. Scale bar = 10 µm (B-F), 5 µm (A). Abbreviations: Ap, apophysis; N, large primary nucleus; R, rhizoid; S, septum; Z, zoospore.

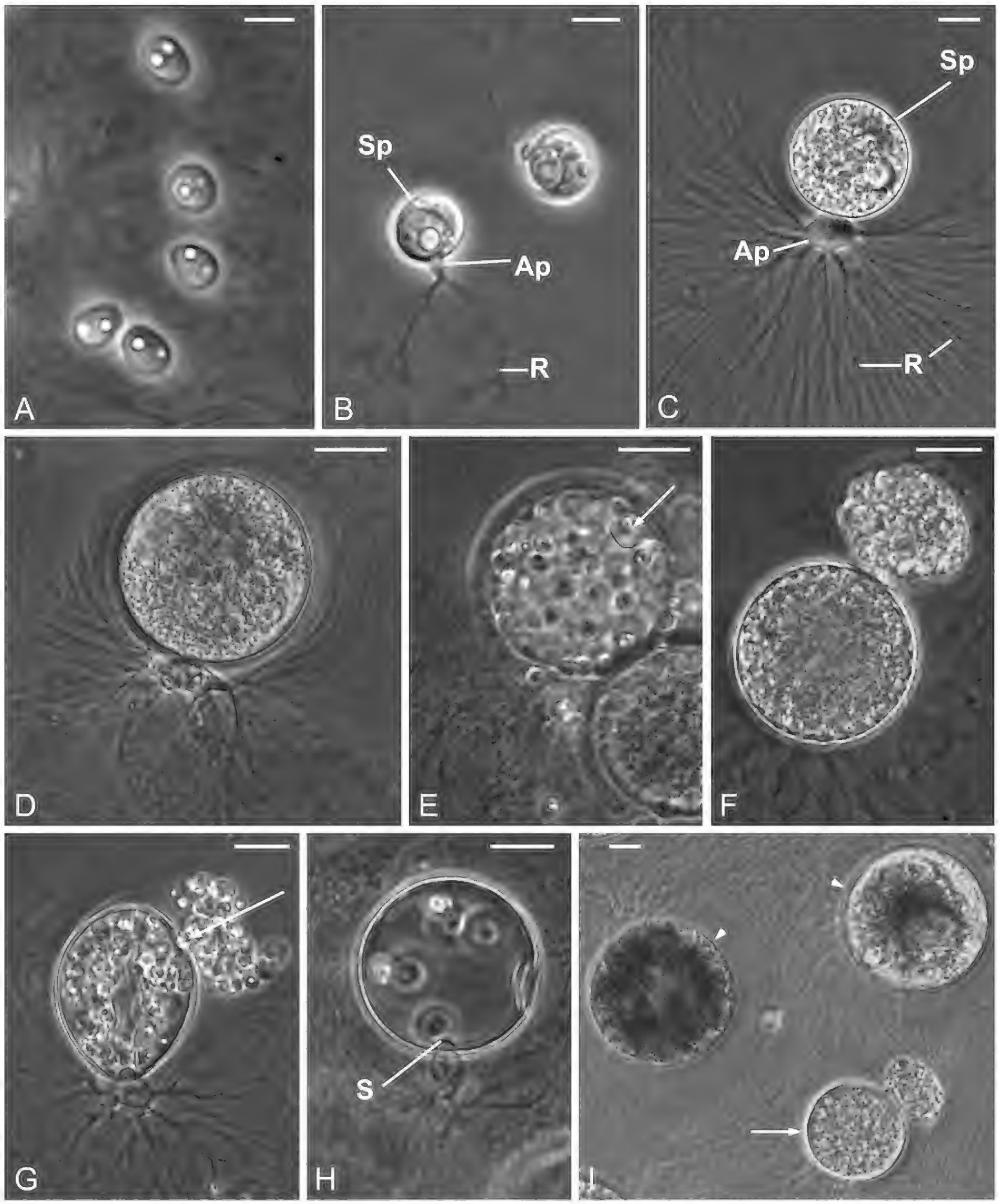

Figure 8. Thallus morphology of *R. pessaminum* sp. nov., strain JEL 823. (A) Motile zoospores containing two lipid globules. (B) Germling with spherical sporangium, a subsporangial apophysis, and rhizoids. (C) Immature sporangium with transversely elongate apophysis. (D) Maturing sporangium. (E) Mature sporangium with cleaved zoospores; arrow indicates location of discharge pore. (F) Vesicular discharge of zoospores. (G) Late in zoospore discharge; arrow indicates location of discharge pore. (H) Empty sporangium, dome-shaped septum, prolate apophysis. (I) Two sporangial sizes indicated; smaller sporangium (arrow) is beginning vesicular discharge of zoospores; larger sporangia (arrowheads) without cleaved contents. Scale bar = 5 µm (A, B), 10 µm (C–I). Abbreviations: Ap, apophysis; R, rhizoid; S, septum; Sp, sporangium.

Table 1. Strains used in phylogenetic analysis

Strain	Location of Collection	Substrate	GenBank 28S Accession #
	Rhizoclosmatium globosum		
JA 20	Vernal Pool, TNF, Hale County, AL[1]	Cellulose	KU721082
JA21	Vernal Pool, TNF, Hale County, AL[1]	Keratin	KU721083
EL100	Lake Lurleen, Coker, AL	Cellulose	JN049527
MB07	Lake Lurleen #2, Coker, AL	Pollen	KC691322
MB10	Lake Lurleen #2, Coker, AL	Chitin	DQ273969
MB37	Lake Lurleen #4, Coker, AL	Chitin	KC691329
MB49	Lake Lurleen #5, Coker, AL	Chitin	*MK328900
WB219	Lake Lurleen, Coker, AL	Chitin	KC691360
WB224	Rainey Pond, Cottondale, AL	Chitin	KC691361
JA5	Lake Nicol, Tuscaloosa, AL	Chitin	KC691316
BR34=	Lake, Gatineau Park, Quebec CANADA	Pollen	JN941008
ATCC2219			
JA2	Lake Nicol, Tuscaloosa, AL	Cellulose	KC691313
JA4	Lake Nicol, Tuscaloosa, AL	Chitin	KC691315
JA18	Vernal Pool,TNF, Hale County, AL[1]	Cellulose	KU721080
JA19	Vernal Pool,TNF, Hale County, AL[1]	Pollen	KU721081
JEL347h	Fen, Perch Pond, Penobscot Co, ME	chitin	DQ273769
JEL863h	Fen, Perch Pond, Penobscot Co, ME	Chitin	KX354826
MB48	Lake Lurleen #5, Coker, AL	Cellulose	KC691131
JEL 800	Fen, Perch Pond, Penobscot Co, ME	Chitin	*MK543211
MP73	Creek culvert, Wagarville, AL	Cellulose	*MK328902
WB236B	Lake Nicol, Tuscaloosa, AL	Chitin	KC691365
WB266A	Broad River, near Bills Mt., Lake Lure, NC	Chitin	*MK328903
WJD111	Lake Lurleen, Coker, AL	Keratin	KC691374
WJD155	Singer Lake Bog, Summit County, OH	Cellulose	*MK328904
WJD205	Vernal Pool, TNF, Hale County, AL[1]	Chitin	KU721085
WJD216	Vernal Pool, Godfrey Drive, Orno, ME	Cellulose	*MK328905
WJD220	Fen, Perch Pond, Old Town, ME	Cellulose	*MK328906
ATCC22918	Camp Powhatan, Pulaski County, VA	Rotifer	*MK328907
JEL 852	Fen, Perch Pond, Penobscot Co, ME	Chitin	*MK328901
	Rhizoclosmatium sparsum, sp. nov		
MP46	Marr's Spring, Tuscaloosa, AL	Pollen	JX905523

MP56	Marr's Spring, Tuscaloosa, AL	Chitin	JX905524
WB266C	Broad River, near Bills Mt., Lake Lure, NC	Chitin	JX905528
NBRC10255	Sugadaira Montane, Research Center, Japan		NBRC10255
	Rhizoclosmatium umbonatum, sp. nov.		
MP44	Wheeler Wildlife Refuge, AL	Pollen	KF257907
WJD185	Vernal Pool,TNF, Hale County, AL[1]	Dragonfly wing	KU721087
	Rhizoclosmatium umbonatum		
	var. *sphaericum,* var. nov.		
JEL128	Lake, Mud Pond, Hancock Co., ME	Cellulose	*MK328908
JEL516	Fen, Perch Pond, Penobscot Co., ME	Chitin	*MK328909
JEL796	Fen, Perch Pond, Penobscot Co., ME	Chitin	*MK328910
	Rhizoclosmatium persicum, sp. nov.		
EL102	Lake Lurleen, Coker, AL	Cellulose	JN049529
MP14	Lake at Lake Lure, NC	Chitin	*MK328914
MP15	Lake at Lake Lure, NC	Chitin	*MK328915
MP67	Lake Nicol, Tuscaloosa, AL	Cellulose	KC691343
WJD187	Vernal Pool,TNF, Hale County, AL[1]	Daphnia	KU721088
	Rhizoclosmatium pessaminum, sp. nov.		
JEL823	Fen, Perch Pond, Penobscot Co., ME	Chitin	*MK328911
JEL849	Fen, Perch Pond, Penobscot Co., ME	Chitin	*MK328913
	Outgroup		
BR97 = ATCC 24931	*Chytriomyces hyalinus*: Ramsayville Marsh, near Ottawa CANADA)	Moribund green alga	AY439074
	1 Oakmulgee District of the Talladege National Forest (TNF)		
	*Indicates newly generated sequences		